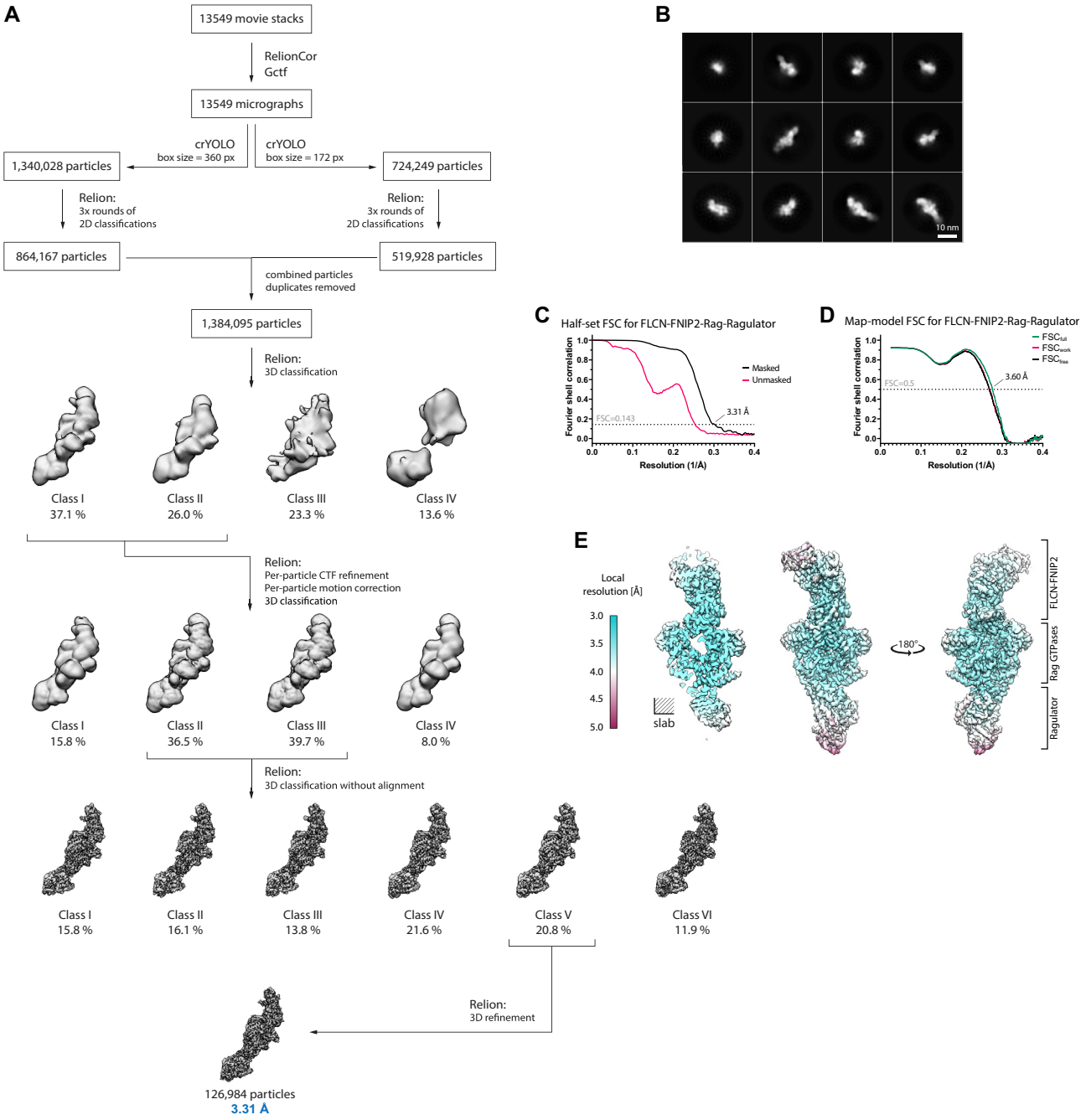

Figure S1. Structural Determination of the FLCN-FNIP2-Rag-Ragulator Complex, Related to Figure 1

(A) Workflow for data processing of the FLCN-FNIP2-Rag-Ragulator dataset.

(B) Sample images for the 2D clustering of the FLCN-FNIP2-Rag-Ragulator complex. Views of the complex from different perspectives can be observed and clustered from the dataset.

(C and D) Half-set gold-standard Fourier shell correlation (FCS) (A) and map-model FSC (B) for the FLCN-FNIP2-Rag-Ragulator.

(E) Local resolution of the FLCN-FNIP2-Rag-Ragulator cryo-EM density map.

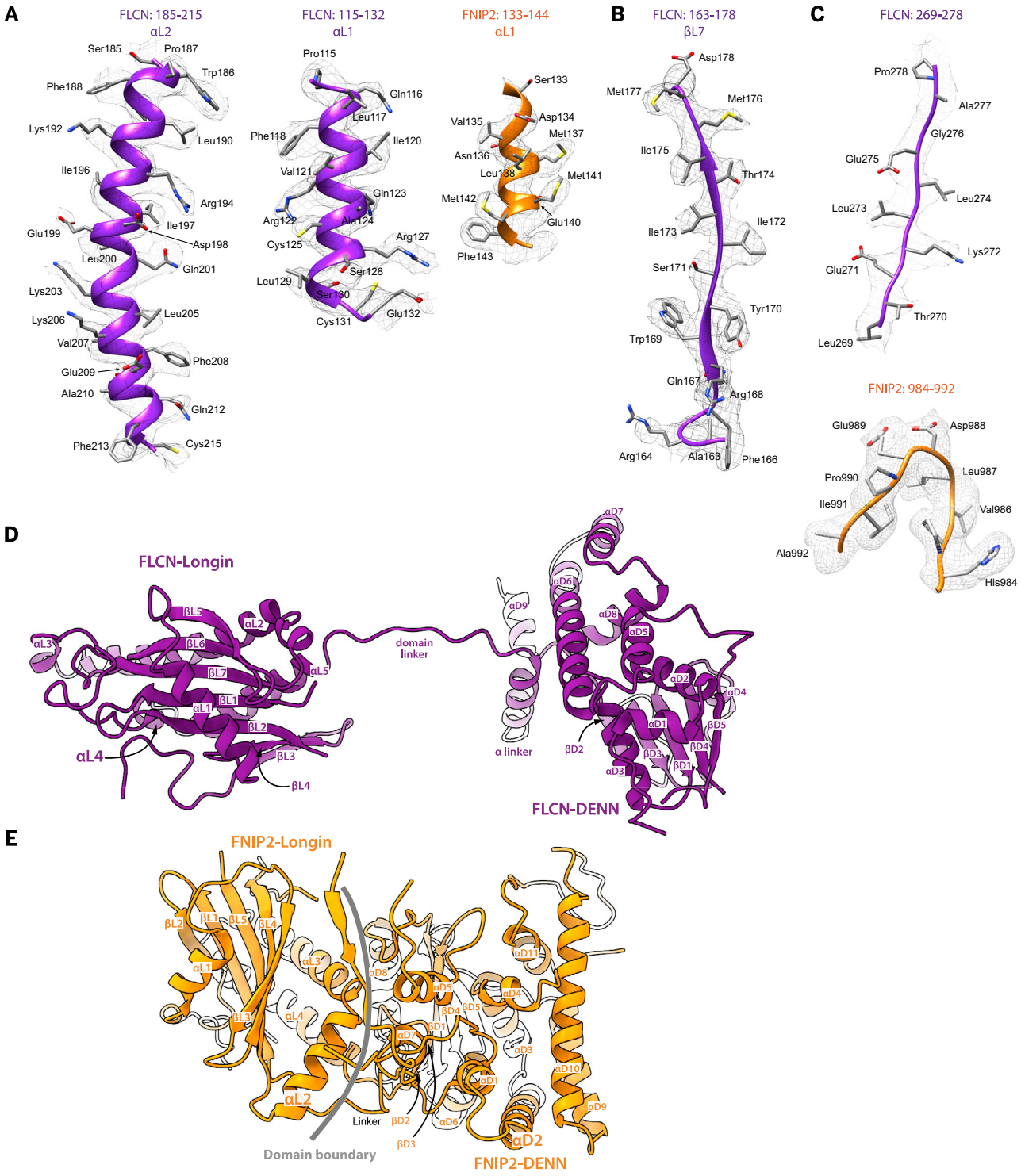

Figure S2. Structural Model for FLCN-FNIP2, Related to Figure 2

(A–C) Sample regions from the cryo-EM density maps and the fitted structural model for α-helical (A), β strand (B), and loop (C) regions of FLCN. Secondary structures and bulky side chains can be registered and resolved at the current resolution.

(D) Architecture of FLCN, domain arrangement, and registration of secondary structure.

(E) Architecture of FNIP2, domain arrangement, and registration of secondary structure.

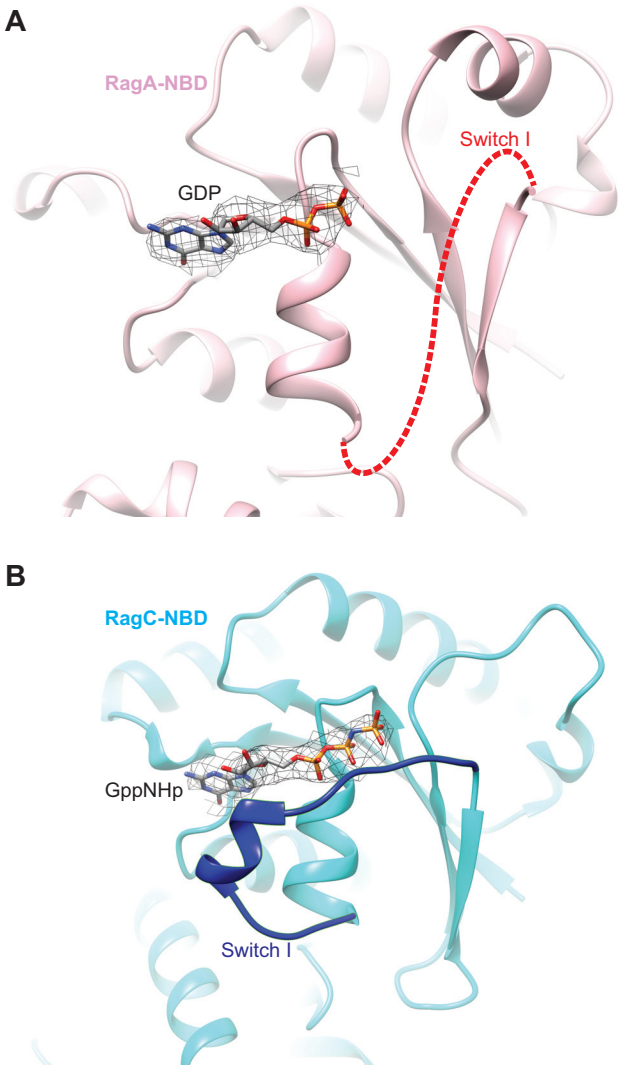

Figure S3. Nucleotide Binding of the Rag GTPases, Related to Figure 3

(A and B) Bound nucleotide and the corresponding cryo-EM density for RagA (A) and RagC (B). Switch I of RagA vanishes as indicated by the dashed line. Switch I of RagC is shown in blue.

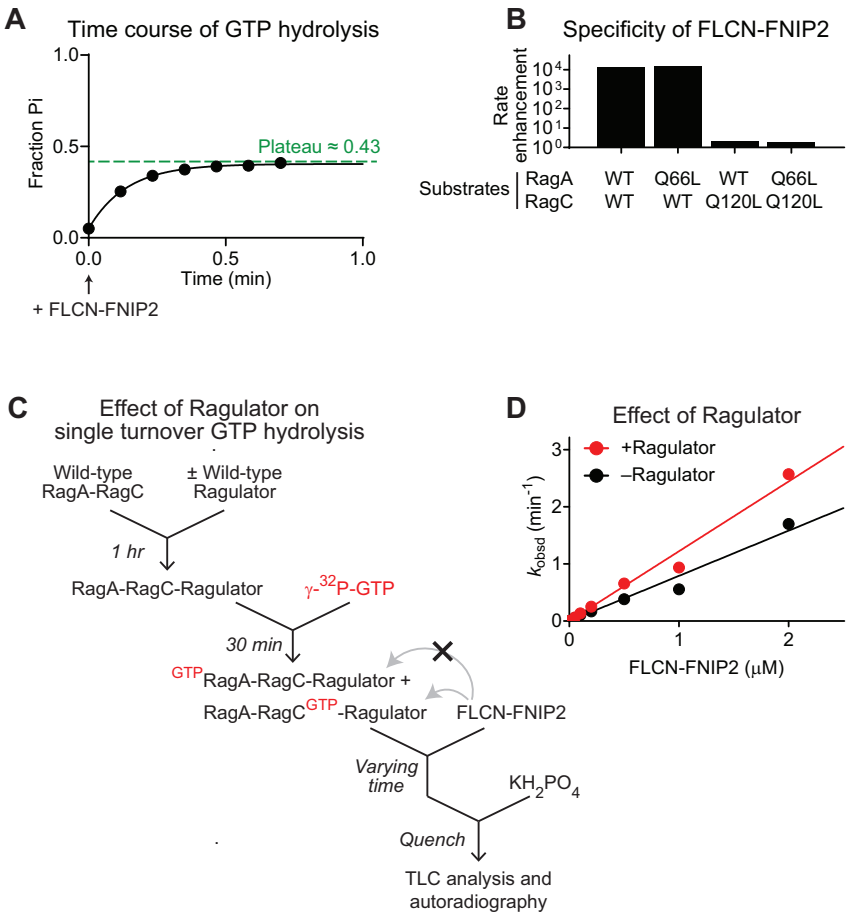

Figure S4. Stimulated Hydrolysis Assay to Probe the Effect of FLCN-FNIP2, Related to Figure 4

(A) Sample time course of a GTP hydrolysis reaction when the reaction was let to reach completion. Only ~50% of the GTP was hydrolyzed, suggesting the bound GTP from only one subunit was stimulated to hydrolyze by FLCN-FNIP2. This experiment was repeated twice and a representative dataset is shown here.

(B) Specificity of GTP hydrolysis reaction using mutant RagA(Q66L) or RagC(Q120L). The Rag GTPase heterodimers carrying the RagC(Q120L) mutation abolish the stimulatory effect of FLCN-FNIP2, while those carrying the RagA(Q66L) mutation maintain it. This experiment was repeated twice and a representative dataset is shown here.

(C) Single turnover GTP hydrolysis assay to determine the influence of Ragulator on the stimulatory effect of FLCN-FNIP2.

(D) Ragulator has mild impact on the stimulatory effect of FLCN-FNIP2. When the Rag GTPases bind Ragulator, FLCN-FNIP2 stimulates GTP hydrolysis to a similar extent as the Rag GTPases alone. This experiment was repeated twice and a representative dataset is shown here.